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The role of moderate static magnetic fields on biomineralization of osteoblasts on sulfonated polystyrene films

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ABSTRACT

We have investigated the effects of moderate static magnetic fields (SMFs) on murine MC3T3-E1 osteoblasts, and found that they enhance proliferations and promote differentiation. The increase in proliferation rates in response to SMFs was greater in cultures grown on partially sulfonated polytstyrene (SPS, degree of sulfonation: 33%) than in cultures grown on tissue culture plastic. We have previously shown that when the degree of sulfonation exceeded a critical value (12%) [1], spontaneous fibrillogenesis occured which allowed for direct observation of the ECM fibrillar organization under the influence of external fields. We found that the ECM produced in cultures grown on the SPS in the presence of the SMFs assembled into a lattice with larger dimensions than the ECM of the cultures grown in the absence of SMFs. During the early stages of the biomineralization process (day 7), the SMF exposed cultures also templated mineral deposition more rapidly than the control cultures. The rapid response is attributed to orientation of diamagnetic ECM proteins already present in the serum, which could then initiate further cellular signaling. SMFs also influenced late stage osteoblast differentiation as measured by the increased rate of osteocalcin secretion and gene expression beginning 15 days after SFM exposure. This correlated with a large increase in mineral deposition, and in cell modulus. GIXD and EDXS analysis confirmed early deposition of crystalline hydroxyapatite. Previous studies on the effects of moderate SMF had focused on cellular gene and protein expression, but did not consider the organization of the ECM fibers. Our ability to form these fibers has allowed us explore this additional effect and highlight its significance in the initiation of the biomineralization process.

1. Introduction

The influence of static magnetic fields (SMFs) on biological systems has been a topic of considerable interest for many years. It is by now well established that SMFs can prevent decrease in bone mineral density [2] and promote the healing of bone fractures [3,4]. Studies on the effects of SMFs have been classified according to the field strength as follows: weak (<1 mT), moderate (1 mT–1 T), strong (1–5 T) and ultra strong (>5 T). Although there have been few reports on the influence weak SMFs on cell function [5], there is substantial evidence indicating that moderate-intensity SMFs (1 mT–1 T) are capable of affecting a number of biological phenomena such as cell proliferation [6], migration [7], orientation [8], and intracellular Ca^{2+} signaling [9]. This range is particularly interesting since it is easily achieved with rare earth magnets at room temperature, and hence easily applied in a number of therapies, especially orthodontic treatments, where electromagnets or superconducting coils cannot be used.

Bone loss associated with aging or pathologies, is an area of increased research, specifically by being focused on understanding the cell–matrix interactions required for promoting biomineralization and bone repair or regeneration. Even though it is well established that application of SMFs offers a promising adjunct therapy for bone healing and regeneration [3,4,10,11], the underlying mechanism of how they affect bone formation is still unclear.

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Experimental studies evaluating the effects of SMFs *in vivo* have limitations since the effect of magnetic field alone on bone formation cannot be isolated. In contrast, *in vitro* studies with osteoblasts allow for a more precise control of environmental factors, and allow identification of factors affected by SMFs. It has been proposed that diamagnetic properties of the cell membrane allow the SMF to be appreciated almost immediately through alteration of the trans-membrane ion flux [12–14]. Since the ECM proteins are also diamagnetic, it was further proposed that the external magnetic field can influence their structure and orientation even before transcription pathways are activated [15]. Yet, since it is difficult to observe the ECM structure *in vitro*, little data exist regarding this aspect [16]. Pernodet et al. [1] firstly showed that proteins, which normally constitute the ECM, could be adsorbed from solution onto partially SPS thin films and self assemble into fibers. Subburaman et al. [17] later demonstrated that only the protein fraction which formed the fiber structures was capable of biomineralizing, thereby providing a biomimic platform for the simultaneous study of the effects of mechanics and morphology of osteoblast cells and their associated ECM. Because use of SPS promotes ECM fibrilogenesis to mimic the actual *in vivo* processes that determine tissue regeneration. We chose this platform to simultaneously investigate the effects of SMFs on cell and ECM organization, maturation, and mechanical properties.

2. Materials and methods

2.1. Static magnetic field exposure system

An aluminum frame was constructed to support two opposing banks of adjacent Neodymium magnets, with a gap of 1 cm. A standard 24-well plate was placed into the frame and the magnetic field was measured using a Bell 640 Incremental Gauss meter. Six wells were measured and the magnetic field at the bottom of each well was 150 mT, and normal to the plane of the dish. The entire assembly was placed in a 37 °C incubator with 5% CO_2. The control samples were also cultured in a 24-well plate, placed 20 cm below the shelf holding the magnet assembly, and where the field was measured to be less than 1G (indicating the earth's magnetic field).

2.2. Surface preparation

Polished 200 μm thick ⟨100⟩ Si wafers were obtained from Wafer World Corporation, West Palm Beach, FL. The surfaces were cleaned with 10% HF solution and Pirhana etch. Sulfonated polystyrene with 33% degree of sulfonation and molecular weight, $M_w = 175$ K ($M_w/M_n < 1.10$), purchased from Polymer Source, Inc. (Dorval, Canada) was dissolved in N, N-dimethylformamide (Sigma–Aldrich, Inc., St. Louis, MO) and spun-cast onto the Si wafers. The film thickness was measured by ellipsometry, and found to be 20 nm. In order to remove residual solvent and adsorb the film to the substrate, the samples were annealed at 170 °C in an oil free vacuum oven at a pressure of 10^{-3} Torr, as described in our previous work [17].

2.3. Cell culture

MC3T3-E1 cells (subclone 4) were purchased from American Type Culture Collection (ATCC) (Manassas, VA) and maintained at 37 °C (5% CO_2, humidified) in α-MEM (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA). Fresh medium was given 3 times per week at 2 or 3 day intervals. Cells were seeded at an initial density of 50,000 cells/cm^2 in 24-well tissue culture plates (BD Biosciences, Franklin Lakes, NJ). For ECM visualization and proliferation assays, cells were seeded at a lower density (2000 cells/cm^2). Differentiation and mineralization were induced by the addition of 50 μg/mL L-ascorbic acid (Sigma, St. Louis, MO), and 2 mM sodium phosphate (Sigma) after 24 h after initial plating (day 1).

2.4. Proliferation assay

The number of cells with or without SMF exposure on day 0, 1, 3, 5 and 7 was determined by counting cell nuclei stained with 4′-6-Diamidino-2-phenylindole (DAPI). Two different types of substrates were used for each group: SPS thin film, and tissue culture plastic (BD Biosciences, Franklin Lakes, NJ). The cell cultures were then imaged using a digital camera attached to the Fluorescent Microscope (Olympus IX51, Center Valley, PA) with 10× objective lens. Fifty images of each culture were imported into Image J (available at http://rsbweb.nih.gov/ij/download.html) for cell counting. Counting was performed in 3 independent cultures for each sample.

2.5. Scanning probe microscopy (SPM) and shear modulation force microscopy (SMFM)

SPM imaging and SMFM measurements were done at room temperature on ECM fibers and living cells using a Dimension 3100 SPM (Veeco, Santa Barbara, CA) in contact mode with a silicon nitride tip (0.06 N/m, Veeco, Santa Barbara, CA). Use of the SMFM to measure shear modulus response relies on lateral modulation of the cantilever (buried ~3 nm deep into the living cell or the ECM fiber) and measurement of the amplitude response, as reported previously [18,19]. At each time point, the sample was measured, in CO_2-independent medium (Invitrogen), with scanning force microscopy in a 35 mm tissue culture dish (BD, Franklin Lakes, NJ). ECM fiber heights were evaluated using SPM cross-sectional images by measuring fiber heights referenced to flat base regions as previously reported by our laboratory [17,20]. For every time point, two different samples were imaged for each time point and 30 fibers on each sample were analyzed. The error bars shown for fiber height data represent the standard deviation obtained from 60 measurements.

2.6. Confocal laser scanning microscopy

MC3T3-E1 cells cultured for 1, 5, 7 days were rinsed with Ca, Mg-free phosphate buffered saline (PBS), fixed with 3.7% formaldehyde (in PBS), and stained with 20 μg/mL propidium iodide (in PBS) for nuclei visualization and a 1% solution of Alexa Fluor 488 Phalloidin (Invitrogen) for F-actin visualization. Cells immersed in PBS were imaged using a Leica Confocal Laser Scanning Microscope with a 63× objective lens.

2.7. Protein assays and gene expression

Alkaline phosphatase (ALP) activity was measured on culture days 1–14 from samples with and without SMF exposure. Cells were washed 3 times with PBS and lysed in the buffer solution (0.1% Triton X-100, 25 mM Tris–HCl). The cells were then scraped off and transferred to microcentrifuge tubes and centrifuged for 30 min at 10,000× *g*. The supernatants were collected and *p*-nitrophenyl phosphate was added as the substrate to react for 2 h at 37 °C. The reaction was stopped with 0.2 N NaOH, and the absorbance at 405nm was determined. The aliquot of the cell lysates was also analyzed for protein content using a Bichinonic acid (BCA) protein assay kit (Sigma–Aldrich, Inc., St Louis, MO). ALP activity was normalized to total protein concentration.

Osteocalcin secretion was measured using the Osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, MA), following manufacturer's instructions. The optical density at 450 nm was measured using a microplate reader. Prior to placing the substrates into the tissue culture dishes, the dishes were blocked by 2% bovine serum albumin (Sigma–Aldrich, Inc., St Louis, MO) to prevent adhesion of cells outside of the substrate (ensures that the osteocalcin source are only cells grown on the substrates). Total protein concentrations were determined as described above and osteocalcin concentrations were normalized to total protein for each time point.

To quantify levels of gene expression, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used. Cells were cultured as described above and at the selected time points (day 14 and day 28) total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was purified according to the manufacturer's instructions. For quantitative PCR, a reference pool was generated by combining equivalent amounts of RNA from all the samples used from the various wells. Primers were custom designed to amplify 150–215 base pair fragments within the coding sequences of osteocalcin (forward primer: 5′-TGAGGACCCTCTCTCTGCTC-3′ and reverse primer: 5′-AGGTAGCGCCGGAGTCTATT-3′) (Invitrogen, Carlsbad, CA). All reactions utilized the One-Step QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) and were performed using a Light Cycler (Roche, Branford, CT). Reaction conditions were optimized for each of the genes studied using an annealing temperature of 57 °C and RNA concentration (10 ng/reaction). Each experimental run consisted of the experimental sample as well as a 5 point serial dilution of the reference pools for both genes. The calculated concentrations for the experimental samples for osteocalcin were normalized to the corresponding total RNA. Each run was replicated 3–4 times.

2.8. Scanning electron microscopy (SEM) and grazing incidence X-ray diffraction (GIXRD)

For SEM and GIXRD, cultures were rinsed three times with PBS. The cells were air-dried following a series of ethanol (30%, 60%, 80%, 90% and 100%) washes. No gold coating was done on the samples for either SEM or GIXRD.

SEM measurements (Helios Nanolab, FEI, Hillsboro, OR) were conducted at the Center for Functional Nanomaterials, Brookhaven National Laboratory (BNL). The morphology of crystals on protein matrices after 28 days was investigated at 15 kV acceleration voltage and 4 mm working distance. Energy dispersive X-ray spectroscopy (EDXS) was used to map the distribution of calcium and phosphate in the ECM fibers and calculate the Ca/P ratio of the minerals.